*Biochem. J.* (1971) **125**, 765–771
*Printed in Great Britain*



# The Effects of the Stress Caused by Experimental Procedures on Alanine, Aspartate, Glutamate and Glutamine in Rat Liver

BY D. F. HEATH, D. R. GEORGE AND J. G. ROSE
*Experimental Pathology of Trauma Section, Medical Research Council Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey, U.K.*

(*Received* 8 *July* 1971)

Rats were stressed by intravenous injection, tail-warming or moderate restraint for 30s, i.e. by stresses imposed by normal handling during experiment. Liver glutamate concentrations were greatly affected. The results were substantially the same in two varieties of rat (Wistar and Sprague–Dawley), in two laboratories, in experiments carried out by two sets of workers, and after all three stresses. The following detailed results refer to Wistar rats. 1. In starved rats at 20°C and 30°C and in post-absorptive rats at 20°C stress by injection raised liver glutamate concentrations from 1.54, 1.57 and 1.88 $\mu$mol/g wet wt. 30s after injection to 3.4, 2.7 and 3.6 $\mu$mol/g wet wt. respectively a few minutes later. In starved rats at 20°C the concentration then fell slowly to 2.3 $\mu$mol/g wet wt., in starved rats at 30°C it remained steady, and in post-absorptive rats at 20°C it rose slowly to about 4.3 $\mu$mol/g wet wt. The final values seemed fairly steady and corresponded to an 'alert' state. 2. In starved rats at 20°C anaesthesia, with or without injection or cannulation during it, raised glutamate concentrations to the 'alert' values, which were maintained for 2–3h. 3. Liver alanine concentration in post-absorptive rats initially fell from 1.5 to 0.8 $\mu$mol/g, and then stayed fairly constant. 4. Aspartate and glutamine concentrations altered only in starved rats, and proportionately much less than those of glutamate. 5. The necessity for knowing the time-dependence of glutamate concentrations after experimental handling is emphasized. 6. There is no wholly satisfactory explanation of the observations.

There have been several reports on the concentrations of alanine, aspartate, glutamate and glutamine in the livers of rats. The effects of starvation were studied by Williamson, Lund & Krebs (1967*a*), Williamson, Lopes-Vieira & Walker (1967*b*), Paleologos, Muntwyler & Kesner (1969) and by Burns, Meghal & Koeppe (1970); of diabetes by Kirsten, Kirsten, Hohorst & Bücher (1961) and by Williamson *et al.* (1967*a*,*b*); of age by de Guglielmone & Gómez (1966); of dosing with phenylalanine by Carver (1965); of ischaemia by Brosnan, Krebs & Williamson (1970); of injury by Heath & Threlfall (1968); and of tumour growth by Wu & Bauer (1960). No-one seems to have considered whether the handling the rat receives in the course of an experiment can alter the concentrations of amino acids. We have shown that handling considerably altered concentrations of glutamate in liver in two varieties of rat in two laboratories with two groups of handlers. Smaller changes were also brought about in the concentrations of alanine, glutamine and aspartate.

## MATERIALS AND METHODS

*Chemicals and enzymes.* L-[U-$^{14}$C]Glutamic acid was from The Radiochemical Centre, Amersham, Bucks., U.K. Analytical Grade resins Dowex AG1 (X8; 200–400 mesh; $Cl^-$ form) and Dowex AG 50W (X2; 300–400 mesh; $H^+$ form) were from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The Dowex AG1 resin was converted into and stored as its acetate form. Glutamate dehydrogenase [L-glutamate–NAD oxidoreductase (de-aminating), EC 1.4.1.2], malate dehydrogenase (L-malate–NAD oxidoreductase, EC 1.1.1.37) and aspartate aminotransferase (L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1) were from Boehringer Corp. (London) Ltd., London W.5, U.K.

*Animals.* Two strains of rat were used: Porton Wistar albino and Sprague–Dawley. Rats were males (220–250g body wt.) fed on M.R.C. diet 41B (Bruce & Parkes, 1956), and kept from shortly after weaning until 2–3h before experiment in animal rooms with constant temperatures (20°C for the Wistar strain, 18°C for the Sprague–Dawley strain) and 12h light per day from 7.00 to 19.00h. Starved rats had had food withdrawn at 9.00h on the preceding day, and were subjected to experiment between 9.30 and

15.00h, after starvation for 24–30h. Post-absorptive rats had had food withdrawn at 9.00h and were subjected to experiment on the same day between 14.00 and 16.00h (see Heath & Threlfall, 1968, for more details of this state).

*Stress.* Three kinds of stress were applied. (1) The rat was held firmly by the scruff of the neck, its tail warmed in water at 48°C for 30s, and 0.2ml of 0.9% NaCl in water (containing usually trace quantities of some labelled material) was injected via a lateral tail vein. This stress lasted 1–3min according to how long injection took. (2) The rat's tail was warmed in water at 48°C for 30s, and the rat was then replaced in its cage, i.e. as (1) above with the injection omitted. (3) The rat was lifted under its belly, placed in a restraining cage for 30s, and then let out into an ordinary cage. The restraining cage stopped the rat turning round, but otherwise allowed considerable movement.

Controls were taken directly from the main storage cages and were killed immediately.

*Liver sampling.* Rats were stunned by a blow on the head and the livers were removed and dropped into liquid $N_2$ (time from stunning 6–10s). Each liver was macerated with ice-cold trichloroacetic acid (5%, w/v, about 6ml/g of liver) in a top-drive macerator. The homogenate was centrifuged at 2000*g* for 20min, the trichloroacetic acid was removed by three extractions with ether, and the aqueous layer was adjusted to pH6–7 with 0.75M-NaOH.

*Separation and analysis of alanine, aspartate, glutamine and glutamate.* Alanine was determined in a portion (0.2–0.4ml) of the extract with an amino acid autoanalyser (Technicon, TSM1, C3 resin).

The separation of the other amino acids was substantially as described by O'Neal & Koeppe (1966), as this method gave specimens pure enough for the determinations of specific radioactivity required for another study. Of the neutralized extract 12ml was concentrated to 4ml in an air or $N_2$ stream in a test tube in a water bath at 45–50°C. The concentrated material was transferred quantitatively to a water-washed column of Dowex AG1 resin (6ml, 240mm long) which was eluted with 30ml of water followed by 0.3M-acetic acid (by the method of Hirs, Moore & Stein, 1954). The first 12ml of aqueous eluate, which contained the glutamine, was collected, as were the fractions at 9–20ml and 30–50ml from the start of the elution with acid. These fractions contained glutamic acid and aspartic acid respectively. The glutamine fraction was acidified, concentrated, and hydrolysed in 3M-HCl for 16h at 105°C. Most of the acid was removed by rotary evaporation, and the product, neutralized, was separated as glutamate by ion-exchange chromatography as described above. The fractions in acetic acid were concentrated at 50°C by an air stream to about 10ml when necessary, and were passed through a column of Dowex AG 50 resin (1ml capacity), which was washed with 20ml of water. The amino acids were eluted with aq. $NH_3$ (1.0M, 5ml; Patel & Balázs, 1970). The $NH_3$ was mostly removed in a stream of air blown on to the solution in a test tube in a water bath at 50°C. One drop of 0.5M-NaOH was then added, and the rest of the $NH_3$ was evaporated off. One drop of 0.5M-HCl was then added to neutralize the solution.

To check recoveries [U-$^{14}$C]glutamic acid was added to the trichloroacetic acid used for protein precipitation. The overall recoveries by the procedure were 98.7±2.6% (±S.D., $n = 6$).

In the normal procedure specimens for chromatography were prepared from liver during one day, stored overnight at 4°C and chromatographed the following day. It was shown, by chromatographing six unconcentrated specimens the day the livers were obtained, that storage and concentration at 45–50°C did not break down glutamine. The glutamine concentrations were not significantly higher than in experiments carried out in the normal way.

Storage of neutralized liver extracts for more than 2 weeks at −20°C caused variable losses of glutamine. In some specimens stored for 4 weeks over half the glutamine had broken down. Glutamate, aspartate and alanine were all stable for at least 3 weeks under these conditions.

Glutamate was determined with glutamate dehydrogenase, aspartate with aspartate aminotransferase and malate dehydrogenase as described by Bergmeyer (1968).

All concentrations are given in μmol/g wet wt.±S.E.M., with the number of rats in parentheses, except where otherwise stated.

## RESULTS

*Glutamate.* The concentrations of glutamate found in the livers of Porton Wistar rats at various times after stress by intravenous injection are shown in Fig. 1. Nearly every point in Fig. 1 summarizes concentrations in rats killed at intervals over several months. There was no correlation between the time of the experiment and glutamate concentration. Least-mean-squares straight lines were fitted to the results as described in the legend to the figure. The 'true' time-dependence is obviously not reproduced by straight lines with abrupt changes of slope, but this treatment does show mean slopes over finite periods as precisely as the results allow.

At 30s after stressing by injection the glutamate concentrations calculated from the regression lines were 1.54±0.03μmol/g, 1.57±0.01μmol/g and 1.88±0.10μmol/g in the livers of rats starved at 20°C and 30°C and of post-absorptive rats at 20°C respectively. In these three conditions, there was then an initial rapid rise in glutamate content at the rates 0.25±0.01, 0.17±0.01 and 0.18±0.02μmol/min per g respectively. In starved rats at 20°C the content fell over the period 15–34min after injection to a fairly steady value of 2.29±0.10 (10), considerably greater than the initial one at 30s. In starved rats at 30°C the initial rise was less and a fairly steady value of 2.68±0.09 (41) was reached by about 7min after injection, again very much higher than that at 30s. In post-absorptive rats at 20°C the initial rapid rise was followed by a slower rise, which continued until at least 30min after injection. These experiments were all carried out by one of us (D.F.H.), mostly with another (J.G.R.) holding the rats.

The effects of strain of rat, laboratory and

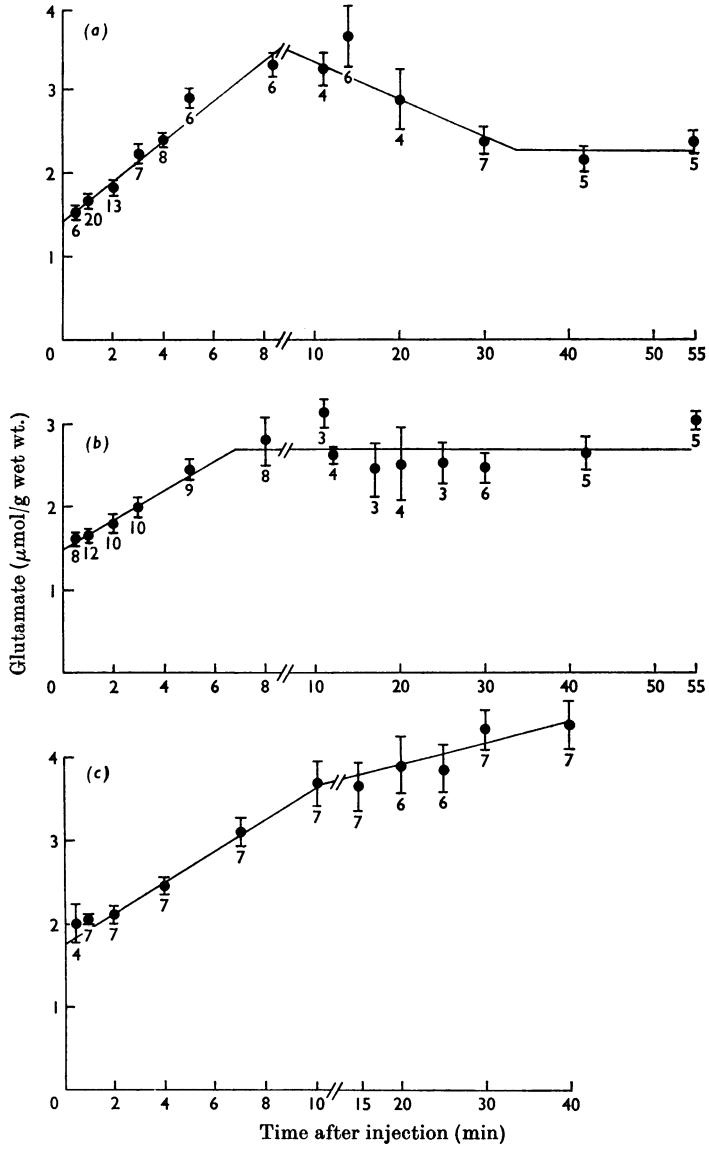


Fig. 1. Variations with time of liver glutamate concentrations in Wistar rats after intravenous injection. Means±S.E.M. are shown, with the numbers in each group below each point. Regression equations are as follows ($y$ is [glutamate]). (*a*), Starved rats, 20°C: 0–8 min, $(y-2.024) = (0.2498\pm0.0099)(t-2.44)$; 8–34 min, $(y-3.064) = (-0.0476\pm0.0140)(t-17.33)$; 34–55 min, $y = 2.285\pm0.104(10)$. (*b*) Starved rats, 30°C: 0–7 min, $(y-1.725) = (0.1724\pm0.0077)(t-1.389)$ 7–55 min, $y = 2.676\pm0.089(41)$. (*c*) Post-absorptive rats, 20°C: 0–10 min, $(y-2.587) = (0.1839\pm0.0194)(t-4.359)$ 10–40 min, $y = 3.625+(0.0256\pm0.0032)t$. The last equation is different in form because to make the lines in Fig. 1(*c*) join, the value for 10 min calculated from the first equation, 3.625, was taken as a fixed point in calculating the second equation.

experimentalist were studied on starved rats at 20°C, and are compared in Table 1 with the results for starved rats at 20°C shown in Fig. 1. The new set of experiments were carried out by D.R.G. either alone or with other colleagues holding the rats. In these experiments the animals for each value were killed within 2–3 weeks of each other. One set of control values was obtained for Wistar

Table 1. *Effects of stress on glutamate concentrations in the livers of starved rats at* 20°C

Means±S.E.M., with numbers of rats shown in parentheses, are shown. The first line consists of results taken from Fig. 1(*a*), except for the values at 30s and 6min, which were calculated from the regression equation. (There were too few results at 30s for a precise estimate.) This experiment was carried out by D.F.H. and J.G.R. The value at 30s from it is also used as the 'control' for the experiment in line 2. All other experiments were carried out by D.R.G. with various colleagues. The controls for these experiments were not stressed (see text for details).

| | | | Concn. of glutamate (μmol/g wet wt.) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Time after stress ... | 0 | 2 | 6 | 8 | 30 | 120 |
| Strain of rat | Laboratory | Nature of stress | (Controls) | | | | | |
| Wistar | A | As Fig. 1(*a*) | 1.54±0.03‡ | 1.82±0.08 (13) | 2.91±0.04 | 3.29±0.14 (6) | 2.39±0.17 (7) | — |
| | A | Tail warmed | 1.54±0.03‡ | 2.12±0.03 (4) | 2.65±0.42 (4) | 2.78±0.10 (4) | 2.48, 2.52* | — |
| | B | Injected | 1.66±0.06 (8) | 2.12±0.03 (4) | 2.34±0.04 (3) | 2.79±0.10 (4) | 2.47±0.04 (4) | — |
| Sprague–Dawley | B | Injected | 2.19±0.01 (7) | 2.17±0.05 (5) | — | 3.64±0.16 (6) | 2.66±0.16 (4) | 2.72±0.08 (4) |
| | B | Tail warmed | 2.19±0.01 (7) | — | — | 3.90±0.33 (5) | — | — |
| | B | Restrained | 2.19±0.10 (7) | 2.17±0.05 (5) | — | 3.59±0.15 (5) | 2.69±0.11 (5) | — |
| Wistar | B | Etherized | 1.66±0.06 (8) | — | — | 2.23±0.06 (4) | — | — |
| | B | Injected under ether | 1.66±0.06 (8) | — | — | 1.91±0.12 (6) | — | — |
| | B | Cannulated under ether, injected via cannula 3h later | 1.66±0.06 (8) | — | — | 2.02±0.08† (6) | — | — |

* These two values were at 24min.
† 8min after injection, 3h 8min after anaesthesia.
‡ 30s after injection.

rats [1.66±0.06 (8) μmol/g] and another for Sprague–Dawley rats [2.19±0.01 (7) μmol/g], and all comparisons in Table 1 for laboratory B are made with these values. The control rats were taken directly from the cages they usually occupied and were stunned immediately. They were, therefore, substantially unstressed before stunning, unlike the rats in the experiments in laboratory A, all of which were stressed. The concentrations in the livers of unstressed Sprague–Dawley rats in laboratory B were significantly higher ($P<0.01$) than in Wistar rats in either laboratory. Injection did not always produce such large increases in starved Wistar rats at 20°C as in the experiment of Fig. 1(*a*) (reproduced in part as the first line of Table 1). The general effects of stress were, however, very similar in all experiments: injection, tail-warming or restraint greatly increased glutamate concentrations within 8min and reversion to normal was incomplete by 30min, or, in the one experiment in which this was studied by 120min (line 4, Table 1). The effects were similar in both laboratories and whichever worker carried out the experiments.

Some attempts were made to lessen the effects of stress on glutamate concentrations in Wistar rats. The results are shown in Table 1. Wistar rats were given light ether anaesthesia for 2–3min. This caused a significant rise in glutamate concentration ($P<0.05$) within 8min of starting anaesthesia. Injection under ether caused an insignificant increase. Other rats were cannulated for 2–3min under ether. A cannula was inserted during this time into a lateral tail vein, and was protected by a lightweight plastic tube attached to the tail by elastic adhesive plaster (Elastoplast). The rats were then kept for 3h and injected via the cannulae. This caused a significant increase over controls. The concentrations reached in all three experiments were very close to the steady concentrations reached after about 30min in other experiments with starved Wistar rats at 20°C (Fig. 1*a* and the last column of the first three lines of Table 1).

*Alanine.* Liver alanine concentrations were only studied in injected post-absorptive Wistar rats at 20°C. There was a marked and highly significant fall ($P<0.01$) in the first 10min, after which the mean values were substantially constant to 40min (Fig. 2). There was, however, marked scatter. There was no correlation after 10min between alanine concentrations and those of glutamate or aspartate.

*Aspartate and glutamine.* These were only studied in Wistar rats stressed by intravenous injection. Most of the results are summarized in Table 2. In the post-absorptive rats both aspartate and glutamine concentrations remained constant during the experimental period, 0.5–40min after injection. In starved rats at both 20°C and 30°C aspartate concentrations were only about 60% as high from 30min after injection as they were in the first 5min after injection. The downward drift between was irregular. The glutamine concentration in starved rats at 20°C drifted downwards roughly linearly over the whole experimental period to about 82% of its initial value, whereas in starved rats at 30°C there was a rapid fall in the first 8min to about 82% of the initial glutamine concentration but this then remained constant within experimental error to the end of the experiment at 55min after injection.

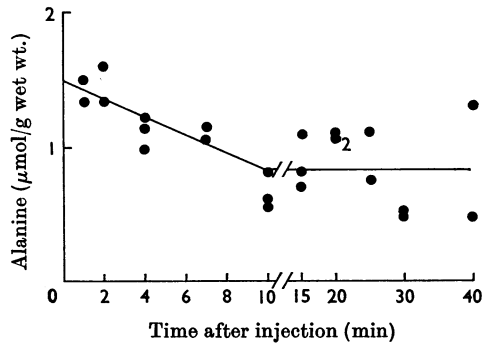


Fig. 2. Variations with time of liver alanine concentrations in post-absorptive Wistar rats at 20°C. Individual values are shown. The regression equation for 1–10min after injection was: $(z-1.085) = -(0.0664\pm0.0081)(t-6.13)$ where $z$ = [alanine]. In obtaining this equation the value at 10min was taken to be 0.826±0.072 (15), its mean value from 10–40min.

## DISCUSSION

The sort of stress that accompanies many experimental procedures altered glutamate concentrations in the livers of rats in ways that were consistent for each nutritional state and environmental temperatures (Fig. 1 and Table 1). One 'stress' was very mild. 'Restraint' did not immobilize the rats, and they were not lifted by their tails. The changes in concentration were very much greater than those reported as brought about by starvation, age, ischaemia, hypophysectomy, injury, tumour growth or dosing with phenylalanine, and were comparable in magnitude with those caused by diabetes (see the introduction for references). The concentrations spanned earlier estimates of normal values. The effects were not much dependent on strain, experimentalist or environment (Table 1), and it is therefore likely that they are general.

Liver glutamate concentrations were inherently

Table 2. *Liver aspartate and glutamine concentrations in Wistar rats injected intravenously*

Means±S.E.M. with numbers of animals in parentheses are given over the stated time ranges in min after injection. In some instances regression equations are given. In these $y$ = amino acid concentrations, $t$ = time in min after injection.

| Rats | Compound | Concn. (μmol/g wet wt.) | Notes and regression equations |
|---|---|---|---|
| Starved, 20°C | Aspartate | 0.5–5 min, 1.032±0.044 (43)<br>30–55 min, 0.635±0.033 (15) | Irregular downward drift between 5 and 30 min |
| | Glutamine | 0.0 min, 5.36±0.15 (from eqn.) | $(y-5.133) = -(0.0180\pm0.077)(t-11.6)$*<br>0.5–55 min |
| Starved, 30°C | Aspartate | 0.5–5 min 1.122±0.049 (27)<br>30–55 min 0.728±0.039 (16) | Irregular downward drift between 5 and 30 min |
| | Glutamine | 0.0 min, 6.34±0.16 (from eqn.)<br>8–55 min, 5.22±0.137 (39) | $(y-5.908) = -(0.1436\pm0.0398)(t-3.04)$<br>0.5–8 min† |
| Post-absorptive, 20°C | Aspartate | 1.061±0.042 (71) | No significant variation with time |
| | Glutamine | 5.73±0.12 (69) | No significant variation with time |

* $P \approx 0.05$, marginally significant.

† Slope more certainly negative than the error on the slope indicates, as 5.22 at 8 min should be regarded as a fixed point.

variable: the coefficients of deviation of the results in Fig. 1 about subset means were 0.19 for starved rats (both groups) and 0.167 for post-absorptive rats. Much greater apparent variability is, however, estimated if one ignores the time-dependence of the effects of stress. For example, by treating all the concentrations in the first 10 min in Fig. 1(*c*) as a single group, the concentration is calculated to be 2.59±0.75 (29) (S.D.), i.e. the coefficient of deviation is 0.29. This is very much greater than the subset deviation of 0.167 ($P \ll 0.01$, F test). Moreover, the observed effects of starvation, for example, would depend markedly on how long after stressing the observations were made. In comparing post-absorptive and starved Wistar rats at 20°C by using the results in Fig. 1 the ratio (±S.E.M.) ([glutamate] in post-absorptive rats)/([glutamate] in starved rats) was 1.26±0.08 at 0 min, 0.96±0.03 at 8 min and 1.92±0.12 at 40 min after injection. The first ratio is greater than unity ($P < 0.01$), the second may not differ from unity, whereas the third is very much greater ($P \ll 0.001$). It is therefore necessary in any experimental procedure involving even mild stress to find the time-dependence of liver glutamate concentrations if meaningful comparisons are to be made.

In starved Wistar rats at 20°C concentrations in unstressed rats (1.66±0.06 μmol/g, Table 1) agreed fairly well with those 30s after injection (1.54±0.03 μmol/g), and it is likely that in other rats the values 30s after injection were fairly close to those before stress, although this has not been proved. What should be regarded as a normal value is, however, debatable. The effects were substantially independent of the apparent severity of the stress. Glutamate concentrations tended towards fairly stable values by 30 min after stress, but these values were higher than those before or shortly after stress (Table 1 and Fig. 1). This suggests that 'stress' did little more than wake the rats up, i.e. the initial and final states were resting and alert. Observations on the behaviour of the rats is in accordance with this view. Rats are nocturnal, and in our experiments were drowsy in their cages until taken out for weighing immediately before the experiment. It is also known that intravenous injection causes an increase in $CO_2$ output from the rat for over an hour (Ashby, Heath & Stoner, 1965; Heath & Stoner, 1968). For planning experiments it is important to note that after anaesthesia liver glutamate was at 'alert' concentrations (Table 1, last 3 lines).

The high net rates of glutamate formation immediately after stress do not seem implausible. Glutamate is formed by the very general transamination reaction: amino acid+2-oxoglutarate → oxo acid+glutamate, and by deamidation of glutamine. Both amino acids and 2-oxoglutarate are supplied considerably faster than the net rate of increase in glutamate, maximally about 0.2 μmol/min per g. 2-Oxoglutarate is formed from citrate at about 1.25 μmol/min per g in rat liver *in vivo* (Sauer, Erfle & Binns, 1970), i.e. about 6 times as fast. In starvation, amino acids, especially alanine and glutamine, supply most of the precursors for gluconeogenesis via glutamate formation (Ross, Hems & Krebs, 1967; Felig, Marliss, Pozefsky & Cahill, 1970; Felig & Wahren, 1971). The gluconeogenic rate required to balance glucose utilization is about 1 μmol/min per g (from Baker, Shipley, Clark, Incefy & Skinner, 1961), equivalent to 2 μmol of precursors/min per g. Glutamate formation from amino acids is probably therefore several times faster than its rate of net increase. In the

post-absorptive rat the rate of transamination cannot be estimated but plasma alanine and glutamine concentrations are at least as high as in starved rats (Paleologos *et al.* 1969), so there is no lack of substrate.

Aspartate and glutamine concentrations fell only in starved rats. Aspartate concentrations fell slowly to about 60% of their values shortly after injection (Table 2). The fractional decrease in liver glutamine was small, but in rats at 30°C was equivalent to the whole net increase in glutamate, and took place over the same period, the first few minutes after injection (Table 2).

There is no satisfactory explanation of the changes observed. The concentrations are interlinked in the cell in a very complex way (see, e.g., Anderson, Nicklas, Blank, Refino & Williamson, 1971; Anderson & Garfinkel 1971), and more work is needed to establish what happens after mild stress.

We are grateful to Miss J. L. Wickens for technical help.

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